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Influence of Body Weight on the Response of Fischer 344 Rats to Anthrax Lethal Toxin

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals on the Institute of Laboratory Animal Resources, National Research Council. The facilitites are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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Groups of Fischer 344 rats were injected intravenously with Bacillus anthracis culture supernatants containing crude anthrax toxin. Times to death of rats given identical toxin preparations varied directly with the weight of the rats (P = Ø.ØØØ1). In contrast to previous reports, the data indicate that rat weight must be taken into account during in vivo assay of anthrax lethal toxin activity.

Bacillus anthracis, the etiological agent of anthrax, possesses two primary virulence factors: a poly-D-glutamic acid capsule (4); and a toxic mixture of three proteins, termed anthrax toxin, consisting of protective antigen (PA), lethal factor (LF), and edema factor (EF) (1). PA and LF together comprise anthrax lethal toxin, whereas PA and EF constitute anthrax edema toxin. Thorne et al. (7) first expressed the toxicity of anthrax toxin in terms of "toxic units" (TU), with the number of TU per ml in a preparation being the reciprocal of the highest dilution giving an edematous response when injected into guinea pig skin. The highest toxic potency described in early studies (1, 3, 7) was 32 TU per ml. For over 20 years, assays of the lethal potency of crude anthrax toxin preparations from various B. anthracis strains, as well as mixtures of purified PA and LF, have been performed by intravenous injection of the material into male, Fischer 344 rats (2, 3, 5, 6). The times to death (TTD) are noted and the toxic potency of the preparations is then determined by using the mathematical formulas and the standard regression line relating TTD to TU described by Haines et al. (3). Those investigators (3) reported that this assay method was valid for rats between 200 and 300 g in weight, with 10 to 12 TU required for lethality and TTD varying insignificantly for rats within this range. In our early studies (2, 5, 6, B. Ivins, unpublished observations), however, TTD in rats injected with crude anthrax toxin appeared to vary directly with weight. Rats weighing 200 g died in substantially less time than 300-g rats which had been injected with identical preparations of toxin. Because assays of lethal toxin or

potency are dependent upon TTD (3), it appeared necessary to determine whether the TTD response is dependent upon rat weight, as our data suggested, or independent of it, as Haines et al. reported (3).

B. anthracis strains Vollum 1B and Sterne were grown in R medium (6) to early stationary phase. Bacteria were removed by centrifugation at 10,000 x g, and the supernatants were filtered and frozen at -70°C in 10-ml amounts. After thawing, 1-ml aliquots of crude toxic supernatants were injected through a 27-Gauge needle into the dorsal penile vein of Fischer 344 rats (Table 1). Rat weight and TTD were recorded. Data from our three, separate experiments and from the experiment reported by Haines et al. (3), were subjected to regression analysis, with TTD plotted against rat weight (Fig. 1). Our data in Fig 1a, 1b, and 1c fit into a linear model, independent of rat or toxin source, with slopes greater than 0 ($P = 0.0001$). The data from Haines et al. (Fig. 1d) did not fit such a model ($P = 0.8069$) and did not demonstrate a slope different from 0. The r^2 values, a measure of the effect of the "weight" variable in reducing variation in the "TTD" variable and thus a measure of the variation in TTD accounted for by the linear model, were 0.756, 0.799, 0.830, and 0.0028 for the data in 1a, 1b, 1c, and 1d, respectively. These values provide additional evidence for the appropriateness of the linear model in describing the data in our three experiments, and they also point to the lack of linearity in the data reported by Haines et al. (3). As further evidence for non-linearity in the previous data (3), the 95% confidence intervals of the mean TTD

for 200-g and 300-g rats, 6.58 min (Table 2), include the mean TTD values for both groups of rats. The data from our three experiments (Table 2) indicate that there is a considerable difference between the TTD of 200-g and 300-g rats injected with identical preparations of anthrax lethal toxin, and that determinations of toxin potency are strongly influenced by rat weight. Our observations differed significantly from those of Haines et al. (3). One possible reason for this may be the large variability in TTD values in the previous data (3), as evidenced by 95% confidence intervals of the mean TTD for 200-g and 300-g rats which were more than three times larger than the corresponding confidence intervals in our three experiments. Another reason may be that the assumption of no significant difference in TTD between 200-g and 300-g rats was based on only 27 data points (3), whereas each of our three regression lines was based on at least 66 data points.

It is clear from comparing our data with the previous data (3) that rat weight must be taken into account when performing in vivo determinations of anthrax lethal toxin activity. Recent studies (2, 5, 6) have demonstrated that the following precautions are essential to minimize TTD variability during anthrax lethal toxin potency determinations: i) Fischer 344 rats of identical weight should be used, especially when comparing the potencies of different toxin preparations; ii) the penile vein must not be ruptured during intravenous injection of the toxin preparation through a 27-Gauge needle; iii) after injection, leakage of the toxin from the injection site should be prevented by application of slight

pressure on the vein at the injection site; and iv) TTD should be measured by a specific parameter, such as cessation of heartbeat.

The rat lethality assay is a rapid method for evaluating anthrax lethal toxin potency. By taking the precautionary measures described above, the assay is also highly accurate and reliable.

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TABLE 1. Fischer 344 rats and anthrax lethal toxin preparations used for correlations of rat weight and time to death

Experiment	Rat Source	Number Used	Weight Range (g)	Source of Toxin[a]
No. 1	M. A. Bioproducts, Walkersville, Md.	88	190 - 436	Vollum 1B
No. 2	Charles River, Kingston, N.Y.	70	196 - 408	Sterne
No. 3	Charles River	66	175 - 410	Vollum 1B
Haines et al.[b] (3)	Fort Detrick, Md.	27	200 - 300	Sterne

[a] Anthrax lethal toxin potency in experiments 1-3 was determined on three separate lots of crude R medium culture supernatants (one from the Sterne strai and two from the Vollum 1B strain of B. anthracis).

[b] Used for comparison by permission from Frederick Klein and the American Society of Microbiology.

TABLE 2. TTD and toxic potency in 200-g and 300-g rats

Experiment	200-g Rats TTD[a]	200-g Rats TU/ml[b]	300-g Rats TTD	300-g Rats TU/ml[c]	ΔTTD[d]	TU_{200}/TU_{300}[e]
No. 1	58.06 (±1.24)	157	66.60 (±0.63)	92	8.54	1.70
No. 2	77.04 (±1.83)	60	91.25 (±1.01)	44	14.21	1.36
No. 3	68.21 (±1.55)	84	80.89 (±0.92)	55	12.68	1.53
Haines et al.[f]	91.06 (±6.58)	44	89.83 (±6.58)	45	1.23	0.98

[a] TTD in minutes (± the 95% confidence interval, predicted from the regression analysis).

[b] Apparent anthrax lethal toxin potency (TU per ml) as determined in 200-g Fischer 344 rats according to the assay method of Haines et al. (3).

[c] Apparent anthrax lethal toxin potency (TU per ml) as determined in 300-g Fischer 344 rats.

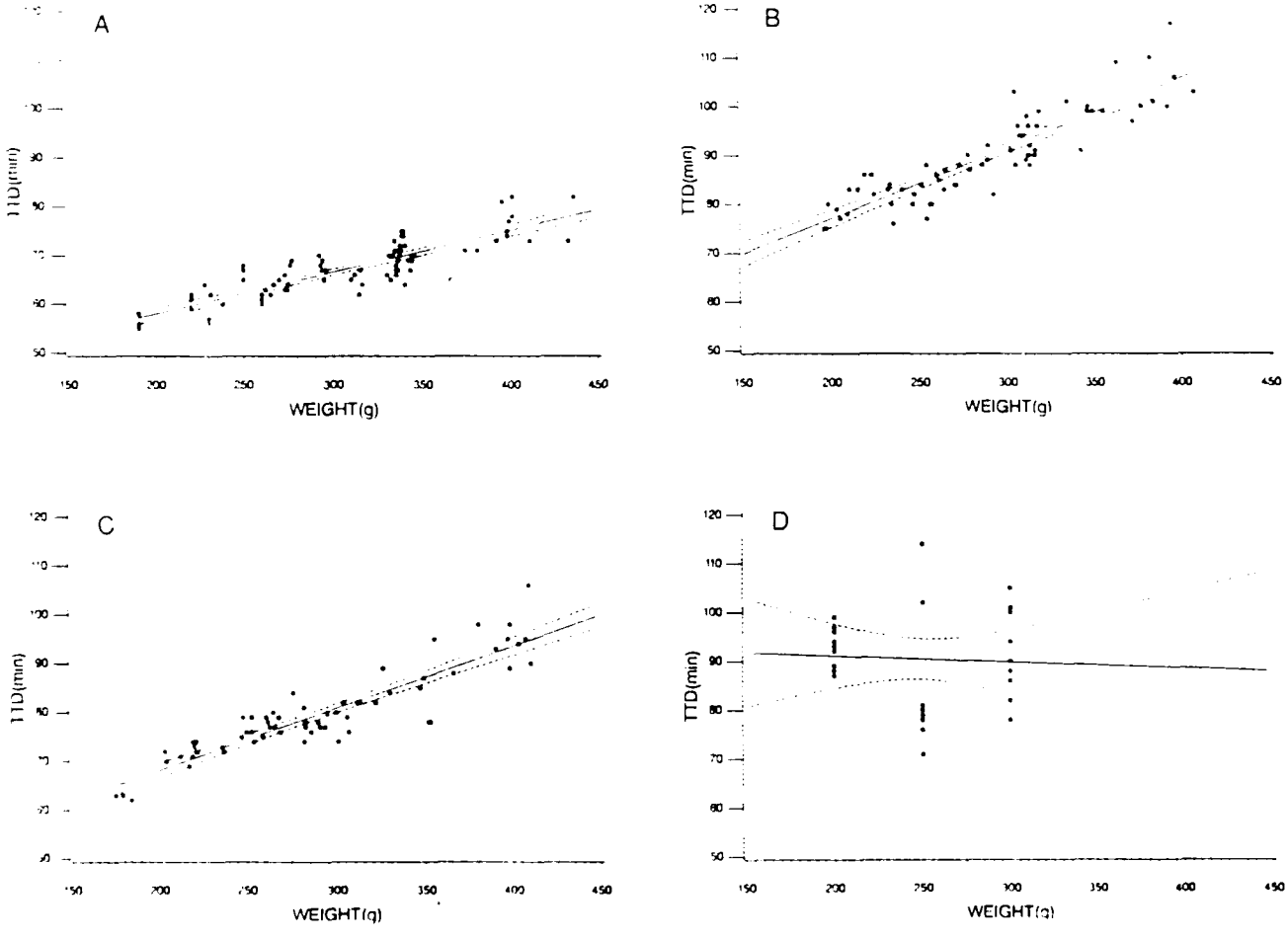
[d] Absolute difference in TTD between 200-g and 300-g rats injected with identical toxin preparations.

[e] Ratio of the toxin potency as measured in 200-g rats to the toxin potency as measured in 300-g rats.

[f] Used for comparison by permission from Frederick Klein and the American Society for Microbiology.

FIGURE LEGENDS

FIG. 1. Regression analysis plots of TTD in minutes versus rat weight in grams. Male, Fischer 344 rats were injected with crude anthrax lethal toxin and TTD were noted. Figures 1a, 1b, and 1c are based on the data from experiments 1, 2, and 3, respectively. Figure 1d is based on the data reported by Haines et al. (3). Broken lines represent the 95% confidence intervals for the mean, predicted TTD values.


A
B
C
D
TTD(min)
WEIGHT(g)
150
200
250
300
350
400
450
50
60
70
80
90
100
110
120

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